# Ceramide Generation in Nitric Oxide-induced Apoptosis: Activation of Magnesium-dependent Neutral Sphingomyelinase via Caspase-3

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**key words: ceramide, nitric oxide, apoptosis, sphingomyelinase, caspase-3**

**Running Title; Ceramide Generation in NO-induced Apoptosis**

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## SUMMARY

**Sodium nitroprusside (SNP), a nitric oxide (NO) donor has been recognized as an inducer of apoptosis in various cell lines. Here we demonstrated the intracellular formation of ceramide, a lipid signal-mediator, in SNP-induced apoptosis in human leukemia HL-60 cells, and investigated the mechanisms of ceramide generation. The levels of intracellular ceramide increased to at most 160 % of the control level in a time- and dose-dependent manner when the cells were treated with 1 mM SNP. SNP also decreased sphingomyelin level to approximately 70 % of the control level and increased magnesium (Mg)-dependent neutral sphingomyelinase (N-SMase) activity to 160 % of the control 2 h after treatment. Neither acid SMase nor Mg-independent N-SMase was affected by SNP. Caspases are thought to be a key enzyme in apoptotic cell death. Acetyl-Asp-Glu-Val-Asp-Aldehyde (DEVD-CHO), a synthetic tetrapeptide inhibitor of caspases inhibited both Mg-dependent N-SMase, ceramide generation, and apoptosis. Moreover, recombinant purified caspase-3 increased Mg-dependent N-SMase in cell free system. These results suggested that the findings that SNP increased ceramide generation and Mg-dependent N-SMase activity via caspase-3, are interesting to the future study to know the relation between caspases and sphingolipid metabolites in NO-mediated signalling.**

## INTRODUCTION

Nitric Oxide (NO) is a highly reactive and unstable free radical gas, which can cross cell membrane easily by diffusion without depending on any release or uptake mechanisms. NO is involved in several signalling pathways related to a diverse array of cell functions. Low levels of NO constitutively produced by an endothelial nitric oxide synthase (NOS), play a physiological role like in regulation of vasodilatation (1) and platelet aggregation (2). On the other hand, high levels of NO produced by an inducible NOS mainly in macrophages and neutrophils, mediate cytotoxicity as the first line of self-defence against invading microorganisms (3) or tumour cells (4). Recently, NO-mediated apoptosis was reported in macrophages (5,6), pancreatic beta cell line (7), and mouse thymocytes (8). NO-generating compounds such as sodium nitroprusside (SNP), S-nitroso-N-acetylpenicillamine (SNAP), and S-nitroglutathione have been reported to induce apoptosis in human leukemia HL-60 and U937 cells (9, 10, 11). Although the mechanisms of NO-mediated cytotoxicity are still controversial, several possible systems described as follows have been proposed; 1) formation of iron-nitrosyl complexes with FeS-containing critical enzymes, which would cause an impairment of mitochondrial function and an energy depletion (12), 2) direct DNA damage by deamination and crosslinking of DNA, which increase mutagenesis (13), 3) generation of peroxynitrite by reaction of NO with superoxide, which may play a significant role in the cytotoxic process (14, 15), and 4) inactivation of several anti-oxidant enzymes, including catalase, glutathione peroxidase, and superoxide dismutases (15, 16).

Sphingolipid metabolites including ceramide have been implicated as potential

regulatory molecules in signal transductions involving cell growth, differentiation and death. Many stresses against cell viability such as TNF-α, anti-Fas antibody, ionizing radiation, serum deprivation (17, 18), anti-cancer drugs (19, 20), heat-shock (21) and hydrogen peroxide (22) were reported to be accompanied with increase of intracellular ceramide. As downstream targets of ceramide, ceramide-activated protein phosphatase (23), protein kinase C (PKC) -ζ (24), a ceramide-activated protein kinase (25), and interleukin-1ß converting enzyme family of proteases called caspases (26) have been suggested. We have also demonstrated the requirement of transcription factor components AP-1 and cytosolic translocation of PKC-δ and -ε from membrane fraction for ceramide-mediated apoptosis (27, 28).

Recently, a family of proteases known as caspases has been implicated as a common executioner of a variety of death signals. Caspase-dependent ceramide generation has been proposed in several apoptosis models (29, 30, 31), whereas Mizushima et al. reported that cell-permeable ceramide induced the cleavage and activation of caspase-3 (32). These results may indicate the activation of caspases both upstream and downstream of ceramide production, but precise relation between caspase cascade and ceramide generation in apoptosis remaines to be clarified. NO was also reported to increase caspase-3 to induce apoptosis (33).

Here, the relation between ceramide generation and caspase-3 in the apoptosis induced by NO in HL-60 cells has been investigated. We demonstrated that SNP, a NO donor increased ceramide generation via activation of magnesium (Mg)-dependent neutral sphingomyelinase (N-SMase), but not via Mg-independent and acid SMase. The

ceramide generation seemed to be mediated upstream of NO-activated caspase, because the inhibition of caspase by acetyl-Asp-Glu-Val-Asp-aldehyde (DEVD-CHO) prevented both NO-induced apoptosis, activation of Mg-dependent N-SMase and ceramide formation. Moreover, recombinant purified caspase-3 could increase N-SMase activity in cell free system. This is the first report suggesting the effect of caspase-3 on Mg-dependent N-SMase as far as we know.

## Materials and Methods

*Cells and Reagents.* Human leukemia HL-60 cells were kindly provided by Dr. M. Saito (National Cancer Institute, Japan). C2-ceramide was purchased from Matreya, Inc.. [ $\gamma$ -$^{32}$P]ATP (6000 Ci/mmol) was purchased from Amersham Corp.. Diacylglycerol (DAG) kinase was kindly provided by Dr. Y. Hannun (Duke University). Sodium-nitroprusside was purchased from E. Merck Darmstadt (Frankfurter Strasse, Germany). Acetyl-Asp-Glu-Val-Asp–$\alpha$-(4-methyl-coumaryl-7-amide)(DEVD-MCA) and DEVD-CHO were purchased from Peptide Institute (Osaka, Japan), dissolved at 10 mM in DMSO and stored at -80 °C. Recombinant purified caspase-3 and -6 were prepared as described before (34). Other chemicals, if not mentioned, were obtained from Sigma Chemical Company (St. Louis, MO.).

*Cell Culture.* Human myelogenous leukemia HL-60 cells were maintained in RPMI 1640 medium containing 10% fetal bovine serum at 37 °C in a 5% $CO_2$ incubator. HL-60 cells in exponentially growing phase were washed in RPMI 1640 media, resuspended in the 2% serum-containing media at a concentration of 2 x $10^5$ cells/ml overnight, and then treated if not described particularly. Viable cell numbers were assessed by 0.025% Trypan Blue dye exclusion method under microscopic observation. Cell numbers and survival rates were also measured by the WST-1 assay (Cell Counting Kit, Dojindo, Japan) using 96-well microplates reader. Reagents were applied 30 min before adding SNP in the culture medium, if not described particularly.

*Analysis of DNA fragmentation.* DNA was isolated by using a GENOME kit (Bio

101, CA), electrophoresed through 3% NuSieve agarose (FMC BioProducts) mini-gel in 1 x TAE (40 mM Tris-acetate, 1 mM EDTA) buffer at 50 V for 3 h and visualized under UV light after ethidium bromide staining.

*Flow Cytometry*. Flow cytometric DNA analysis was performed for quantification of cell death by apoptosis. Due to DNA degeneration and subsequent leakage from cells (35), apoptotic cells can be detected by diminished staining with DNA-specific fluorochromes. In brief, 2 x $10^6$ cells were harvested, washed with PBS, and resuspended in PBS containing 0.5% paraformaldehyde and 0.5% saponin for fixation of cells (36). Then the cells were washed and resuspended in fluorochrome solution (50 $\mu$g/ml propidium iodide, 1 mg/ml RNase (Bachem California, USA)). The red fluorescence was measured with FACScan (Becton Dickinson, USA). We could assess the number of hypodiploid cells (apoptotic cells) and cells with more than diploid DNA content (nonapoptotic cells).

*Ceramide Measurement*. Extraction of cellular lipids by Bligh-Dyer method and ceramide measurement using DAG kinase were performed as described (37, 38). The solvent system to separate ceramide phosphate was chloroform/acetone/methanol /acetic acid/$H_2O$ (10:4:3:2:1). We confirmed that DAG kinase activity was not increased by SNP because C2-ceramide did not change as an internal standard during the procedure, and that amounts of phospholipid phosphate were corresponding to the viable cell numbers (data not shown).

*Nitrite Assay*. NO undergoes a series of reactions with several molecules present in biological fluids. The final products of NO in vivo are nitrite and nitrate. The sum of nitrite and nitrate can be the index of total NO production. Nitrite, a stable NO

oxidation product, was determined using the Griess reaction (Nitrate/Nitrite Assay Kit, CAYMAN CHEMICAL, USA.). Phenol red-free DMEM media were harvested after treatment. Firstly, nitrate was converted to nitrite utilizing nitrate reductase, and then Griess reagents were added for converting nitrite into a deep purple azo compound. The absorbance of azo chromophore was measured to determine a nitrite concentration at 540 nm by using the plate reader.

*Sphingomyelin (SM) Quantitation.* The cells were washed with PBS, seeded at 5 x $10^5$ cells/ml in RPMI media containing 2 % FCS and labelled with [$^{14}$C]choline chloride (0.1 $\mu$Ci/ml) at 37 °C in 5 % $CO_2$ for 36 h. The labelled cells were treated with 1 mM SNP for the indicated times. After harvesting the cells, the lipids were extracted by Bligh-Dyer method, and applied on silica Gel 60 TLC plate (Whatman). Inorganic phosphate in the extract was measured to calculate phospholipid content. TLC plate was developed in the solvent containing chloroform/methanol/acetic acid/$H_2O$ (50: 30: 8: 5) and the bands corresponding to SM were detected by BAS system (Fuji Photo Film, Japan).

*Assay method for SMases.* HL-60 cells (1 x $10^7$) were harvested, washed twice with ice-cold PBS, and homogenized in 0.5 ml lysis buffer containing 10 mM Tris/HCl (pH 7.5), 1 mM EDTA, and 0.1 % Triton X-100 after each treatment, and the homogenate was centrifuged at 100,000 x g for 1 h at 4 °C. The supernatant was used as an enzyme source. The assay mixture for the measurement of Mg-dependent N-SMase contained 0.1 M Tris/Hcl (pH 7.5), 60 nmol of [methyl-$^{14}$C] sphingomyelin (Specific activity; 1.74 Gbq/mmol, Amersham Corp.), 10 mM $MgCl_2$, 0.1% Triton X-100 and 50-300 $\mu$g of enzyme in 0.1 ml of final volume. For Mg- independent N-SMase, $MgCl_2$ was removed

from the reaction mixture. For acid SMase, 0.1 M sodium acetate (pH 5.0) was used instead of Tris/HCl. Incubation was carried out at 37 °C for 30 min. The reaction was stopped by adding 1.5 ml of chloroform/methanol (2:1). Then 0.2 ml of double distilled water was added to the tubes and vortexed. The tubes were centrifuged at 1,000 x g for 5 min to separate the two phases. The clear upper phase (0.4 ml) was removed, placed in a glass scintillation vial, and were counted by scintillation counter (Packard, USA). Protein concentrations were determined by using the Protein Assay kit (Bio-Rad, USA).

*Fluorometric assay of DEVD-MCA cleavage activity.* The cells after each treatment were homogenized in lysis buffer containing 10 mM HEPES/KOH (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM dithiothreitol (DTT), 1 mM PMSF, 100 $\mu$M pepstatin, 0.15 U/ml aprotinin, and 50 $\mu$g/ml leupeptin, and centrifuged at 10,000 x *g* for 10 min. The supernatant was collected as an enzyme source and added to the reaction mixture (10 % sucrose, 10 mM Hepes/KOH (pH 7.4), 5 mM DTT, 0.1 % CHAPS, and 10 $\mu$M DEVD-MCA) which was followed by incubation at 25 °C for 60 min. Fluorescence was measured by a microplate reader (MTP-100F, CORONA ELECTRIC, Japan) using 360 nm excitation and 450 nm emission filters. Concentrations of 7-amino-4-methyl-coumarin (AMC) liberated as a result of cleavage were calculated comparing with standard AMC solutions.

*Preparation of cell extracts for assay of SMase in cell free system.* The cells were suspended in lysis buffer containing 10 mM HEPES/KOH (pH 7.4), 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 1 mM PMSF, 100 $\mu$M pepstatin, 0.15 U/ml aprotinin, and 50 $\mu$g/ml leupeptin, left on ice for 20 min and passed through 27G needle, then centrifuged at 10,000 x *g* for 15 min. Protein concentrations were determined with the BioRad assay.

## RESULTS

*SNP-induced Apoptosis in HL-60 Cells*

SNP showed a time and dose-dependent induction of apoptosis in HL-60 cells (Fig. 1, A and B). Three h after treatment with 1 mM SNP, HL-60 cells showed morphological changes (blebbing, shrinkage, and chromatin condensation) and DNA fragmentation characteristic for apoptosis (Fig. 1C). The percentage of apoptotic cells measured by flow cytometric analysis increased from 8.6% to 30.5% 4 h after treatment with 1 mM SNP. At higher concentrations, the number of apoptotic cells did not show any more increase and necrosis was observed judging from Trypan Blue dye staining. After 24 h the cell numbers decreased to about 20% of the control level (data not shown). Potassium hexacyanoferrate, which is structurally similar to SNP except for the absence of a nitroso group, did not affect cell growth at the same concentration as SNP (data not shown), suggesting that the effects of SNP on cell growth and apoptosis were due to NO generation but not cyanoid effects.

*Increase of Intracellular Ceramide by SNP*

To date, our studies and others have demonstrated that ceramide, a lipid second messenger play an important role in regulating cell growth, differentiation and death (17, 18, 37, 38). To investigate the interrelation between NO and ceramide generation, we measured nitrite concentration in culture media after addition with C2-ceramide. But we could not detect any change within 6 h, while SNP increased nitrite production in a time-dependent manner (Fig.2). Although ceramide was reported to enhance the expression of inducible NOS in rat astrocytes (39), it may not be the case in HL-60 cells

for ceramide to increase NO generation. For examining the possible involvement of ceramide signal pathway in NO-induced cell stress, we measured intracellular ceramide levels after addition of SNP. Ceramide generation measured by DAG kinase assay method began to increase from 90 min after addition of 1 mM SNP, and reached at a maximum level, which was about 160% of the control level, 4 h after treatment (Fig. 3A). To justify ceramide measurement by DAG kinase assay, we confirmed that the activities of DAG kinase and phospholipid phosphate of the same numbers of cells did not change during the treatment with SNP as described in "Materials and Methods." Higher concentrations than 1 mM SNP did not increase ceramide levels more than the effect of 1 mM SNP probably due to the induction of necrosis (Fig. 3B).

*Sphingomyelin Hydrolysis through Mg-dependent N-SMase by SNP*

Since there are several possible metabolic pathways as a mechanism of ceramide generation, we, first of all, measured changes of labelled SM contents by SNP to examine the possible involvement of SMase. SM levels decreased to 70 % of the control level 2 h after treatment with 1 mM SNP and then returned to the control level by 6 h (Fig. 3C). We examined the activities of three different types of SMases, which have been reported to be involved in SM hydrolysis (37). As shown in Fig. 3D, Mg-dependent N-SMase (basal specific activity was 1.08 nmol/mg protein/h) increased to 156±18 % of the control level 2 h after treatment with SNP, and returned to the control level by 6 h. Activity of Mg-independent N-SMase (basal specific activity was 0.65 nmol/mg protein/h) did not change following treatment with SNP up to 6 h. Activity of acid SMase (basal specific activity was 9.72 nmol/mg protein/h) slightly decreased but not

increased after treatment with SNP. The biological meaning of this decrease is unclear at present. These results suggest that ceramide generation by SNP results from SM hydrolysis via the increase of Mg-dependent N-SMase activity.

*Increase of DEVD-MCA Cleavage Activity and its Involvement in SNP-induced Apoptosis*

Since many stresses are reported to activate caspase-3 as an executioner of apoptosis, we investigated whether SNP-generated NO activates caspase-3. The activity of caspase-3 was assessed by measuring the proteolytic cleavage of DEVD-MCA, a fluorogenic substrate of caspase-3, and increased after treatment with SNP (Fig. 4A). The activity 4 h after treatment with 0.5 and 1 mM SNP were 88 and 134 pmol/mg protein/min, respectively, compared to 30 pmol/mg protein/min of the control level. The activities of caspase-3 increased by SNP in a time-dependent manner. DEVD-CHO (200 $\mu$M) an inhibitor of caspase-3 completely inhibited the increase of DEVD-MCA cleaving activity by SNP (Fig. 4B). Moreover, SNP-induced apoptotic cells markedly decreased from 31 % to 11 % by addition of DEVD-CHO (Fig. 4C). These results suggest that the activation of caspase-3 is required to induce HL-60 cell apoptosis by SNP.

*Inhibitory Effects of DEVD-CHO on Ceramide Generation and Activation of Mg-dependent N-SMase*

We next tried to know whether ceramide generation by Mg-dependent N-SMase was upstream or downstream of caspase-3 in SNP-induced apoptosis. We examined the effects of DEVD-CHO on ceramide generation and increase of Mg-dependent N-SMase

activity induced by SNP. SNP-induced increase of intracellular ceramide, which showed the maximum 4 h after treatment was completely inhibited by preincubation with 200 $\mu$M DEVD-CHO as shown in Fig. 5A. By the same procedure, activation of Mg-dependent N-SMase (146±16 % of the control level 4 h after treatment with SNP) was also completely inhibited (Fig. 5B). These results showed that protease including DEVDase is activated upstream of ceramide generation by Mg-dependent N-SMase for NO-induced apoptosis.

*Activation of Mg-dependent N-SMase by purified Caspase-3 in Cell Free System*

It became clear that caspase inhibitor DEVD-CHO inhibits ceramide generation and Mg-dependent N-SMase activity in the process of NO-induced apoptosis. While it is in the nature of things that SMase can generate ceramide through hydrolysis of SM, there was no direct evidence that caspase-3 can increase the activity of Mg-dependent N-SMase. Therefore, we used recombinant purified caspase-3 from E.coli transfected with cDNA of caspase-3, which did not have any types of SMase activity in itself (data not shown), but which could increase Mg-dependent N-SMase activity with the cell extracts. Addition of recombinant purified caspase-3 (600 ng) to the cell extracts (250 $\mu$g) induced 3 fold increase of Mg-dependent N-SMase activity (Fig.6). Since we previously showed that caspase-3 activated caspase-6 in Fas-induced apoptosis (40), we examined whether caspase-6 increased Mg-dependent N-SMase activity, but the activity of that did not increase even in the presence of the cell extracts (data not shown). Our results suggested the direct role of caspase-3 in activating Mg-dependent N-SMase and ceramide generation in NO-induced apoptosis.

## DISCUSSION

The sphingomyelin cycle and ceramide generation were first discovered in cell differentiation of HL-60 cells in response to 1α, 25-dihydroxyvitamin $D_3$ (37, 38) and recent studies have shown that many cell types respond to diverse stresses with ceramide generation (17, 18). Recently, NO has been reported to be related to induction of apoptosis in various cell lines including human leukemia HL-60 cells (5-11). In this report we have investigated whether and how NO is related with ceramide generation in caspase/apoptotic death pathway in HL-60 cells.

First of all, we showed that SNP induced growth inhibition and apoptosis in a time- and dose-dependent manner (Fig.1). We investigated whether ceramide increased NO generation and vice versa. We found that ceramide had no effect on NO generation in HL-60 cells by measuring nitrite in the culture media (Fig.2). This result agree with the previous report showing that ceramide itself did not affect NOS in isolated pancreatic islets and astrocytes, even though ceramide enhanced lipopolysaccharide-induced NOS activation (39, 41). When apoptosis was induced by NO, intracellular levels of ceramide increased about 160% of the control level 4 h after treatment. Since there were several possible mechanisms to generate ceramide metabolically, the changes of SM levels and three different types of SMase were investigated. As shown in Fig. 3C, SM levels decreased 2 to 4 h after treatment with SNP and then returned to the control level, corresponding to the time course of ceramide generation reciprocally. Although acid SMase was reported to be involved in TNF-α-induced apoptosis (42), the activities of

acid SMase and Mg-independent N-SMase did not increase in NO-induced apoptosis (Fig. 3D). These findings showed that NO increases ceramide generation by activating Mg-dependent N-SMase.

Although well-characterized downstream signals of NO are guanylate cyclase (43), adenyl cyclase (44, 45) and protein kinase C (PKC) (46), it is controversial whether cGMP-dependent protein kinase (PKG), cAMP-dependent protein kinase (PKA) and PKC are closely involved in NO-induced apoptosis (47-49). We therefore examined the effects of several kinases, such as PKA, PKG, or PKC on NO-induced ceramide generation by using activators and inhibitors of these kinases. The results demonstrated that neither activators nor inhibitors of PKG, PKA and PKC had any significant effect on ceramide generation (data not shown), suggesting that NO induced ceramide generation, independently of PKA, PKG, or PKC-related signalling.

In the present work, we suggested that ceramide might be an mediator in NO signalling pathway and is generated from SM hydrolysis through the activation of Mg-dependent N-SMase. The membrane-associated neutral Mg-dependent SMase was purified and characteirzed very recently (50). In this study, N-SMase was activated by caspase-3 because DEVD-CHO, a caspase-3 inhibitor blocked both SNP-induced SMase activation and ceramide generation (Fig. 4 and 5). To confirm the more direct effect of caspase-3 on SMase, recombinant purified caspase-3 was added to the cell extracts. In this cell free system we could detect the increase of Mg-dependent N-SMase activity in the presence of recombinant caspase-3 (Fig. 6), while recombinant caspase-6 could not increase SMase activity (data not shown). Judging from the present data, NO-generated ceramide signal is downstream target of caspase-3.

However, at present, in terms of the mode of action of caspases on SMase, it is unknown whether caspase-3 cleaved plausible pro-SMase, cleaved and inactivated SMase inhibitor or activated SMase activator. As long as we surveyed this is the first evidence showing the activation of Mg-dependent N-SMase via caspase-3, while activation of acid SMase activity has shown to occur in response to caspases (51). In terms of the relation of caspase-cascade and ceramide in induction of apoptosis, many previous reports suggest that ceramide is upstream of caspase-cascade (26). Our data and others related to REAPER in *Drosophila* (29), which showed caspase could enhanced ceramide generation *in vivo*, may suggest a new function of ceramide as a modulator of caspase-cascade. At present, unfortunately neither this idea nor the indispensability of caspase-generated ceramide in NO-induced apoptosis can be demonstrated because we do not have any biochemical tool to inhibit directly the activity of N-SMase to generate ceramide.

On the contrary to our data, exposure to NO donors such as SNAP and SNP, or activation of NOS was reported to inhibit apoptosis in T lymphocytes and human umbilical vein endothelial cells (52, 53), because NO induced cGMP-dependent or direct inhibition of caspase-3 through protein S-nitrosylation. These data seem inconsistent with our data showing that NO could induce apoptosis by increasing ceramide generation through caspase-3 activation. The discrepancy of NO effect on apoptosis may be due to the differences of intensity, duration of NO exposure and kinds of cells. Indeed, we observed that the low concentrations of SNP less than 100 $\mu$M showed the protective effect against serum deprivation-induced apoptosis in HL-60 cells. Induction of apoptosis and increase of intracellular ceramide were generally observed by the

treatment with more than 250 $\mu$M SNP. Caspase-3 activity measured by cleavage of DEVD-MCA, was also enhanced at higher, but not lower concentrations of SNP (unpublished data by Takeda and Okazaki).

NO could generate peroxynitrite by reacting with superoxide anion. NO could also modulate endogenous antioxidant enzymes such as catalase, glutathione peroxidase, and superoxide dismutases (15, 16). Since intracellular ceramide were reported to increase by hydrogen peroxide (22), changing of redox status may be another one of the mechanisms regulating apoptotic signals between NO and ceramide generation.

Finally, it remains to be elucidated in the future how NO activates caspase-3 and what are the mechanisms of activation of Mg-dependent N-SMase to understand the biochemical and physiological implications of ceramide signal in NO-induced apoptosis.

## REFERENCES


1. Knowles, R.G., and Moncada, S. (1992) *Trends. Biochem. Sci.* **17**, 399-402

2 Hawkins, D.J., Meyrick, B.O., and Murray, J.J. (1988) *Biochim. Biophys. Acta.* **969**, 289-296

3 Adams, L.B., Hibbs, J.B.-Jr., Taintor, R.R., and Krahenbuhl, J.L. (1990) *J. Immunol.* **144**, 2725-2729

4 Stuehr, D.J., and Nathan, C.F. (1989) *J. Exp. Med.* **169**, 1543-1555

5 Albina, J.E., Cui, S., Mateo, R.B., and Reichner, J.S. (1993) *J. Immunol.* **150**, 5080–5085

6 Sarih, M., Souvannavong, V., and Adam, A. (1993) *Biochem. Biophys. Res. Commun.* **191**, 503-508

7 Ankarcrona, M., Dypbukt, J.M., Bröne, B. and Nicotera, P. (1994) *Exp. Cell Res.* **213**, 172-177

8 Fehsel, K., Kröncke, K.D., Meyer, K.L., Huber, H., Wahn, V. and Kolb-Bachofen, V. (1995) *J. Immunol.* **155**, 2858- 2865

9 Messmer, U.K. and Bröne, B. (1995) *Biochem. J.* **319**, 299-305

10 Kuo, M-L., Chau, Y-P., Wang, J-H., and Shiah, S-G. (1996) *Biochem. Biophys. Res. Commun.* **219**, 502-508

11 Jun, C-D., Park, S-J., Choi, B-M., Kwak, H-J., Park, Y-C., Kim, M-S., Park, R-K. and Chung, H-T. (1997) *Cellular Immunol.* **176**, 41-49

12 Stadler, J. Curran, R.D., Ochoa, J.B., Harbrecht, B.G., and Hoffman, R.A. (1991) *Arch Surg.* **126**, 186-191

13 Nguyen, T., Brunson, D., Crespi, C.L., Penman,B.W., Wishnok, J.S., and Tannenbaum, S.R. (1992) *Proc. Natl. Acad. Sci. USA.* **89**, 3030-3034

14 Lin, K-T., Xue, J-Y., Nomen, M., Spur, B. and Wong, P.Y-K. (1995) *J. Biol. Chem.* **270**, 16487-16490

15 Dobashi, K., Pahan, K., Chahal, A., and Singh, I. (1997) *J. Neurochem.* **68**, 1896–1903.

16 Asahi, M., Fujii, J., Suzuki, K., Seo, H.G., Kuzuya, T., Hori, M., Tada, M., Fujii, S., and Taniguchi, N. (1995) *J. Biol. Chem.* **270**, 21035-21039

17 Hannun, Y.A. (1994). *J. Biol. Chem.* **269**, 3125-3128

18 Mathias, S., Pena, L.A., and Kolesnick, R.N. (1998) *Biochem. J.* ***335***, 465-480

19 Strum, J.C., Small, G.W., Pauig, S.B., and Daniel, L.W. (1994) *J. Biol. Chem.* **269**, 15496-15497

20 Bose, R., Verheij, M., Haimovitz-Friedman, A., Scotto, K., Fuks, Z., and Kolesnick, R. (1995). *Cell.* **82**, 405-414

21 Chang, Y., Abe, A., and Shayman, J.A. (1995) *Proc. Natl. Acad. Sci. USA.* **92**, 12275-12279

22 Verheij, M., Bose, R., Lin, X.H., Yao, B., Jarvis, W.D., Grant, S., Birrer, M.J., Szabo, E., Zon, L.I., Kyriakis, J.M., Haimovitz-Friedman, A., Fuks, Z., and Kolesnick, R.N. (1996) *Nature.* **380**, 75-79

23 Wolff, R.A., Dobrowsky, R.T., Bielawska, A., Obeid, L.M., and Hannun, Y.A. (1994) *J. Biol. Chem.* **269**, 19605-19609

24 Lozano, J., Berra, E., Municio, M.M., Diaz-Meco, M.T., Dominguez, I., Sanz, L., and Moscat, J. (1994) *J. Biol. Chem.* **269**, 19200-19202

25 Liu, J., Mathias, S., Yang, Z., and Kolesnick, R.N. (1994) *J. Biol. Chem.* **269**, 3047–3052

26 Smyth, M.J., Perry, D.K., Zhang. J., Poirier, G.G., Hannun, Y.A., and Obeid, L.M. (1996) *Biochem. J.* **316**, 25-28

27 Sawai, H., Okazaki, T., Yamamoto, H., Okano, H., Takeda, Y., Tashima, M., Sawada, H., Okuma, M., Ishikura, H., Umehara, H. and Domae, N. (1995) *J. Biol Chem.* **270**, 27326-27331

28 Sawai, H., Okazaki, T., Takeda, Y., Tashima, M., Sawada, H., Okuma, M., Kishi, S., Umehara, H. and Domae, N. (1997) *J. Biol. Chem.* **272**, 2452-2458

29 Pronk, G.J., Ramer, K., Amiri, P., and Williams, L.T. (1996) *Science.* **271**, 808-810

30 Dbaido, G.S., Perry, D.K., Gamard, C.J., Platt, R., Poirier, G.G., Obeid, L.M., and Hannun, Y.A. (1997) *J. Exp. Med.* **185**, 481-490

31 Genestier, L., Prigent, A-F., Paillot, R., Quermeneur, L., Durand, I., Banchereau, J., Revillard, J.P., and Bonnefoy-Berard, N. (1998) *J. Biol. Chem.* **273**, 5060-5066

32 Mizushima, N., Koike, R., Kohsaka, H., Kushi, Y., Handa, S., Yagita, H., and Miyasaka., N. (1996) *FEBS lett.* **395**, 267-271

33 Brockhaus, F., and Bröne, B. (1998) *Exp. Cell Res.* **238**, 33-41

34 Takahashi, A., Hirata, H., Yonehara, S., Imai, Y., Lee, K.K., Moyer, R.W., Turner, P.C., Mesner, P.W., Okazaki, T., Sawai, H., Kishi, S., Yamamoto, K., Okuma, M., and Sasada, M. (1997) *Oncogene.* **14**, 2741-2752

35 Nicoletti, I., Migliorati, G., Pagliacci, M.C., Grignani, F. and Riccardi, C. (1991) *J. Immunol. Methods.* **139**, 271-279

36 Yoneda, T., Omori, K., Ishida, A., Uchida, S. Mori, T., Kannagi, R. (1994) *Jpn. J. Med. Tech.* **43**, 1045-1051

37 Okazaki, T., Bell, R.M. and Hannun, Y.A. (1989) *J. Biol. Chem.* **264**, 19076-19080

38 Okazaki, T., Bielawska, A., Bell, R.M. and Hannun, Y.A. (1990) *J. Biol. Chem.* **265**, 15823-15831

39 Pahan, K., Sheikh, F.G., Khan, M., Namboodiri, A.M.S., and Singh, I. (1998) *J. Biol. Chem.* **273**, 2591-2600

40 Hirata, H., Takahashi, A., Kobayashi, S., Yonehara, S., Sawai, H., Okazaki, T., Yamamoto, K., and Sasada, M. (1998) J. Exp. Med. **187**, 587-600.

41 Kwon, G., Bohrer A., Han, X., Corbett, J.A., Ma, Z., Gross, R.W., McDaniel, M.L., and Turk, J. (1996) *Biochem. Biophys. Acta.* **1210**, 308-316

42 Santana, P., Pena, L.A., Haimovitz-Friedman, A., Martin, S., Green, D., McLoughlin, M., Cordon-Carbo, C., Schuchman, E.H., Fuks, Z. and Kolesnick, R. (1996) *Cell* **86**, 189-199

43 Schmidt, H.H.H.W., Lohmann, S.M., and Walter, U. (1993) *Biochim. Biophys. Acta.* **1178**, 153-175

44 Duhe, R.J., Nielsen, M.D., Dittman, A.H., Villacres, E.Cl, Choi, E-J., and Storm, D.R. (1994) *J. Biol. Chem.* **269**, 7290-7296

45 Wang, S., Yan, L., Wesley, R.A., and Danner, R.L. (1997) *J. Biol. Chem.* **272**, 5959–5965

46 Gopalakrishna, R., Chen, Z. H., and Gundimeda, U. (1993) *J. Biol. Chem.* **268**, 27180-27185

47 Loweth, A.C., Williams, G.T., Scarpello, J.H.B., and Morgan, N.G. (1997) *FEBS lett.* **400**, 285-288

48 Nishio, E., Fukushima, K., Shiozaki, M. and Watanabe, Y. (1996) *Biochem. Biophys. Res. Commun.* **221**, 163-168

49 Messmer, U.K., Lapetina, E.G. And Bröne, B. (1995) *Mol. Pharmacol.* **47**, 757-765

50 Liu, B., Hassler, D.F., Smith, G.K. Weaver, K. and Hannun, Y.A. (1998) *J. Biol. Chem.* **273**, 34472-34479

51 Schwander, R., Wiegmann, K., Bernardo, K., Kreder, D. And Kröncke, M. (1998) *J. Biol. Chem.* **273**, 5916-5922

52 Mannick, J.B., Miao, X.Q., and Stamler, J.S. (1997) *J. Biol. Chem.* **272**, 24125–24128

53 Dimmeler, S., Haendeler, J., Nehls, M., and Zeiher, A.M. (1997) *J. Exp. Med.* **185**, 601- 608

**Fig.1**

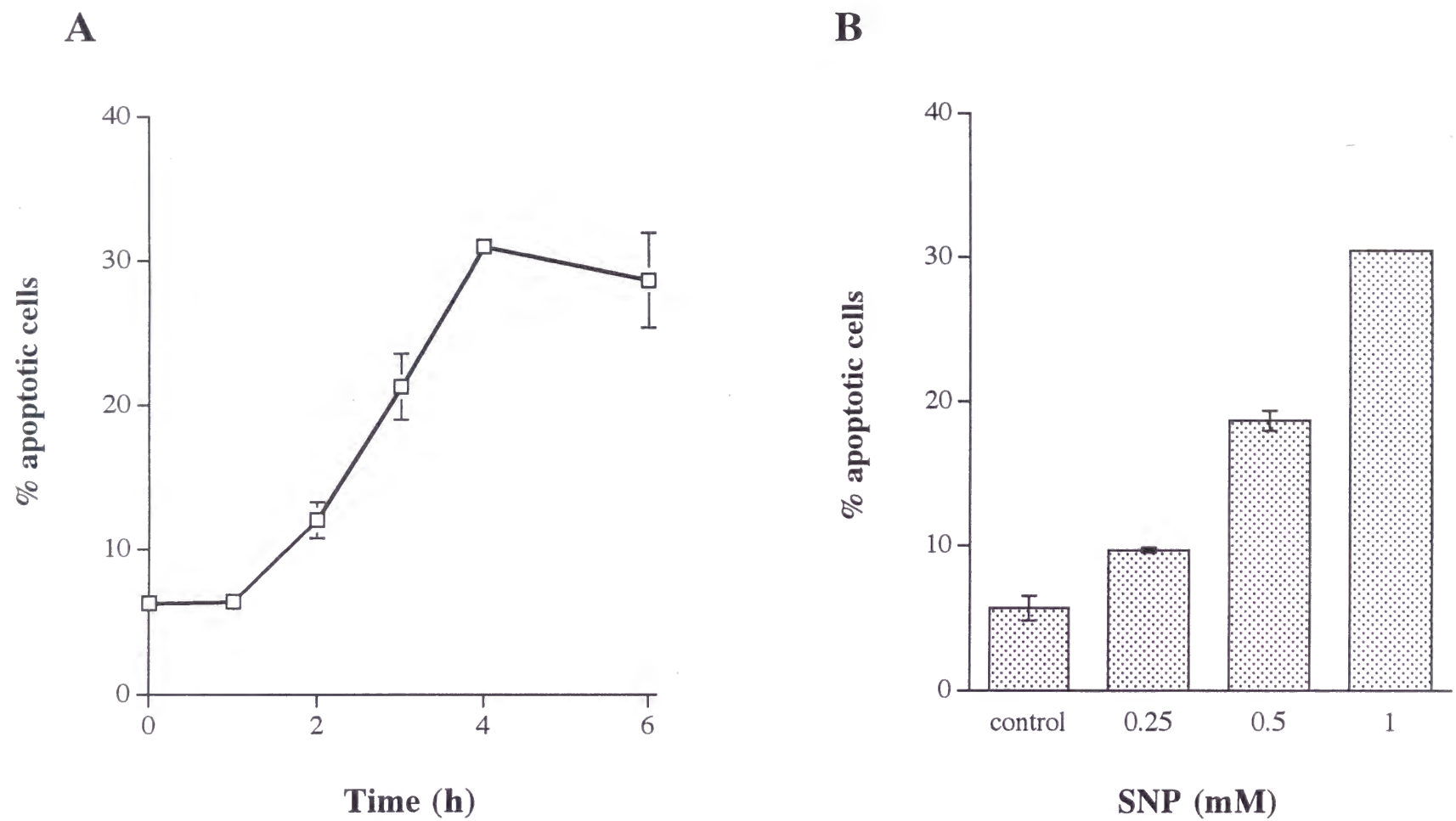

A
% apoptotic cells
40
30
20
10
0
0
2
4
6
Time (h)
B
% apoptotic cells
40
30
20
10
0
control
0.25
0.5
1
SNP (mM)

Fig.1C

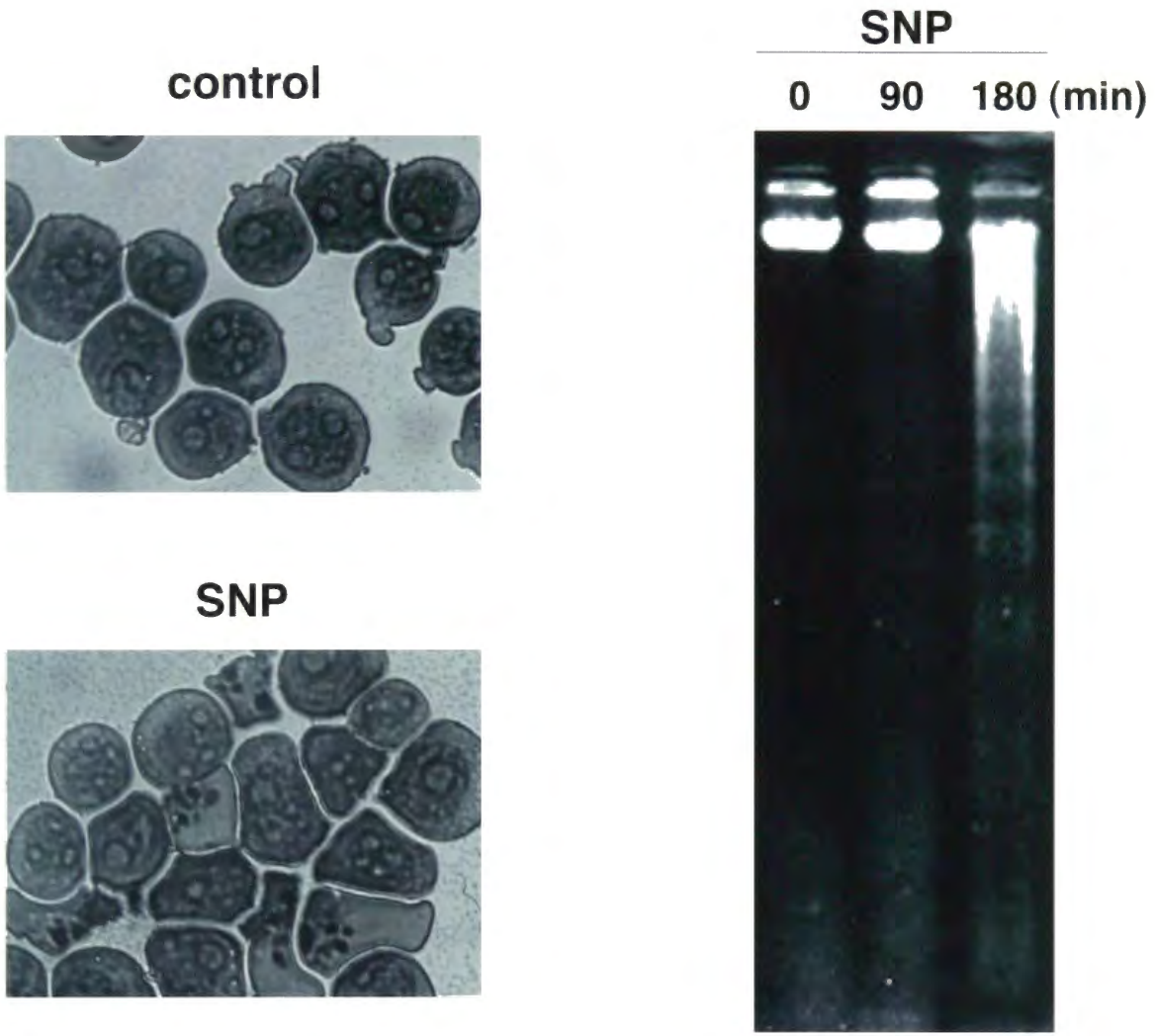


**Fig.2**

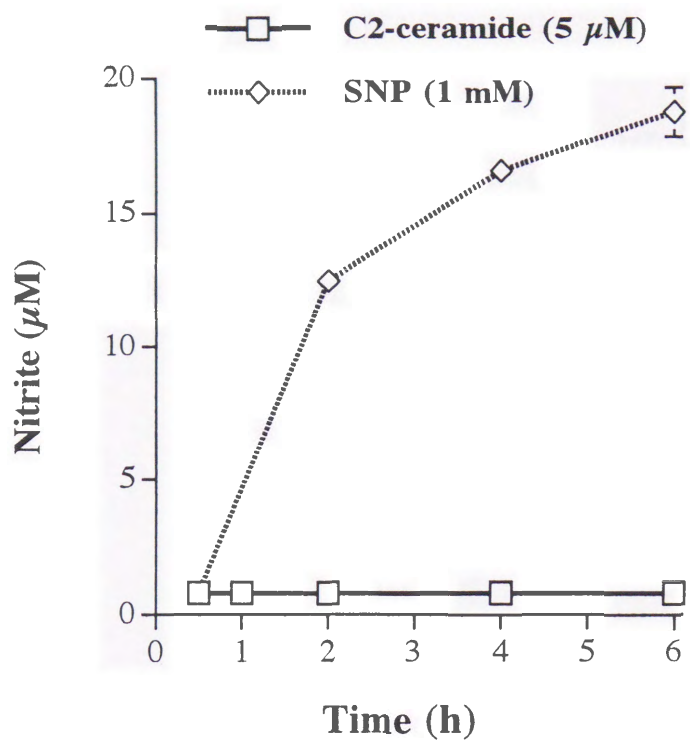


Fig.3

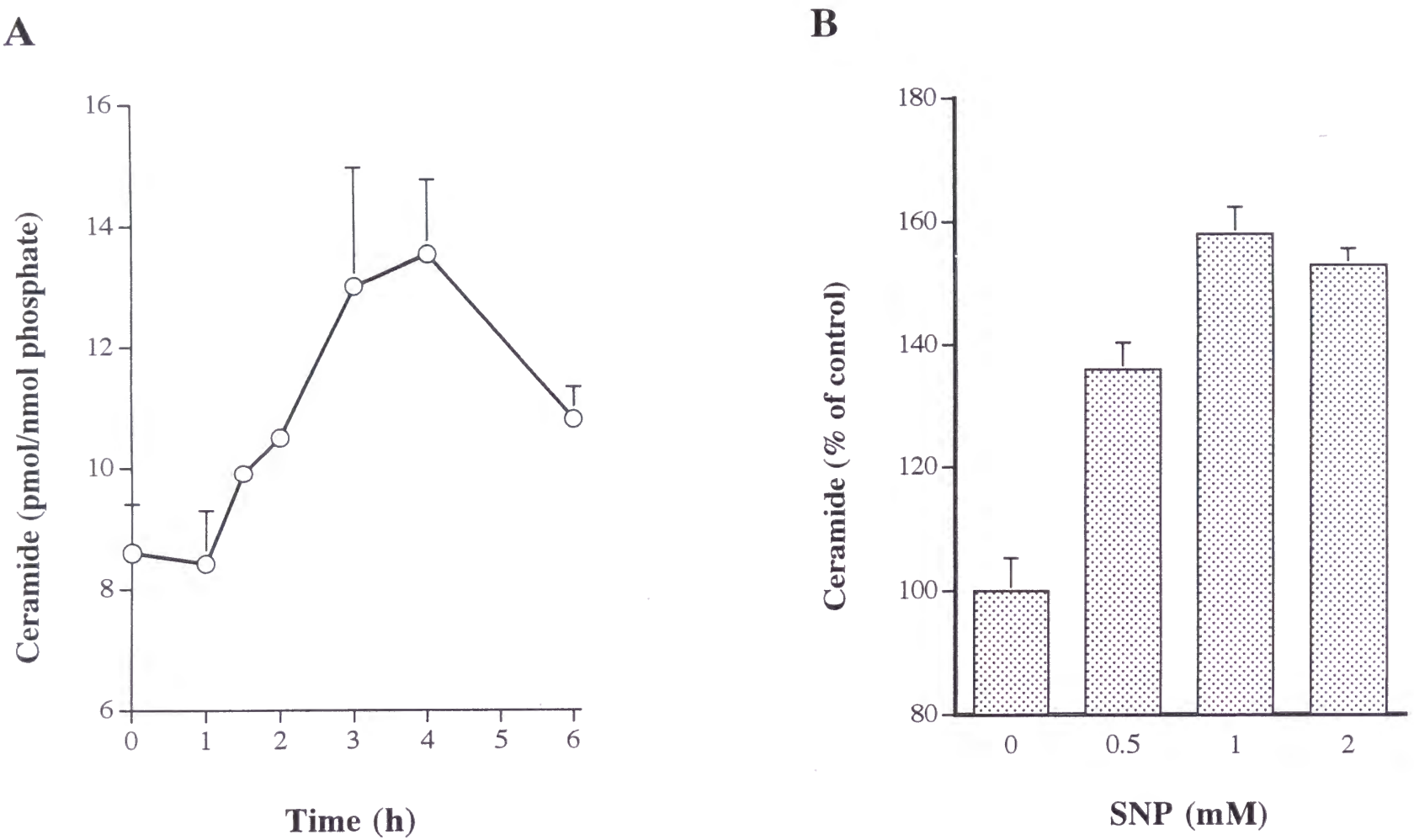
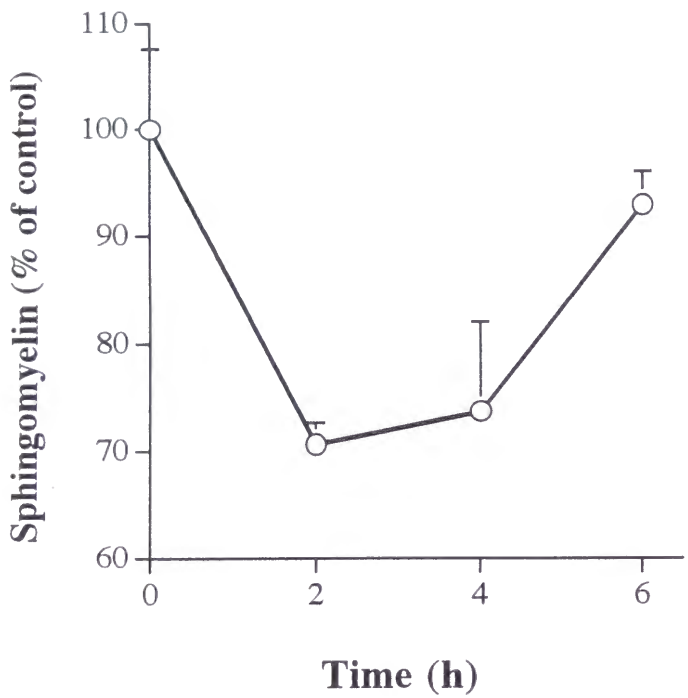

A
Ceramide (pmol/nmol phosphate)
16
14
12
10
8
6
0
1
2
3
4
5
6
Time (h)
B
Ceramide (% of control)
180
160
140
120
100
80
0
0.5
1
2
SNP (mM)

Fig.3

C


Sphingomyelin (% of control)
110
100
90
80
70
60
0
2
4
6
Time (h)

D

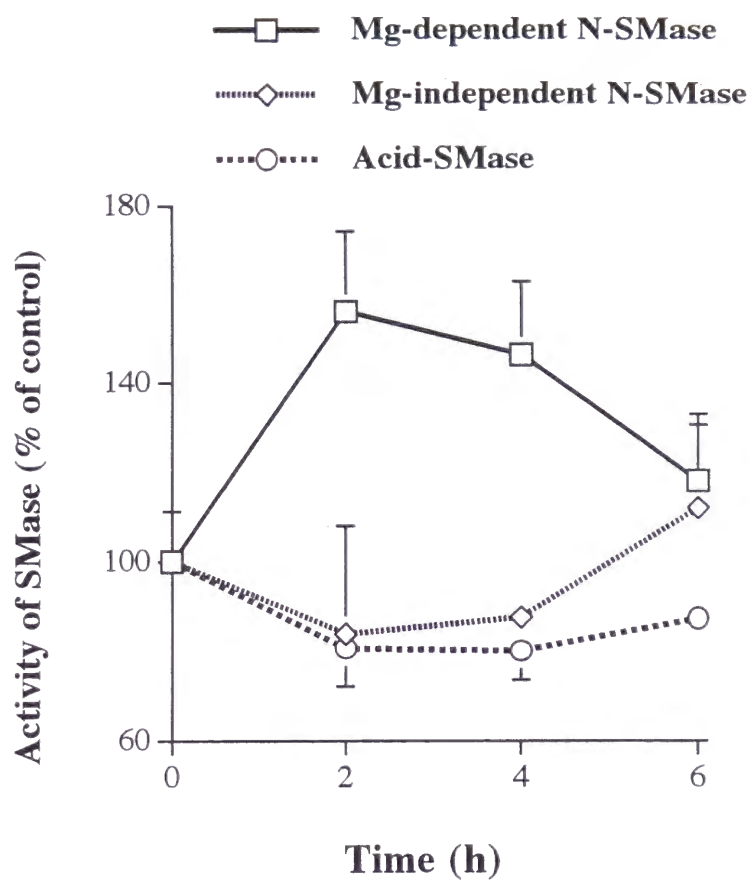

Mg-dependent N-SMase
Mg-independent N-SMase
Acid-SMase
Activity of SMase (% of control)
180
140
100
60
0
2
4
6
Time (h)

Fig.4

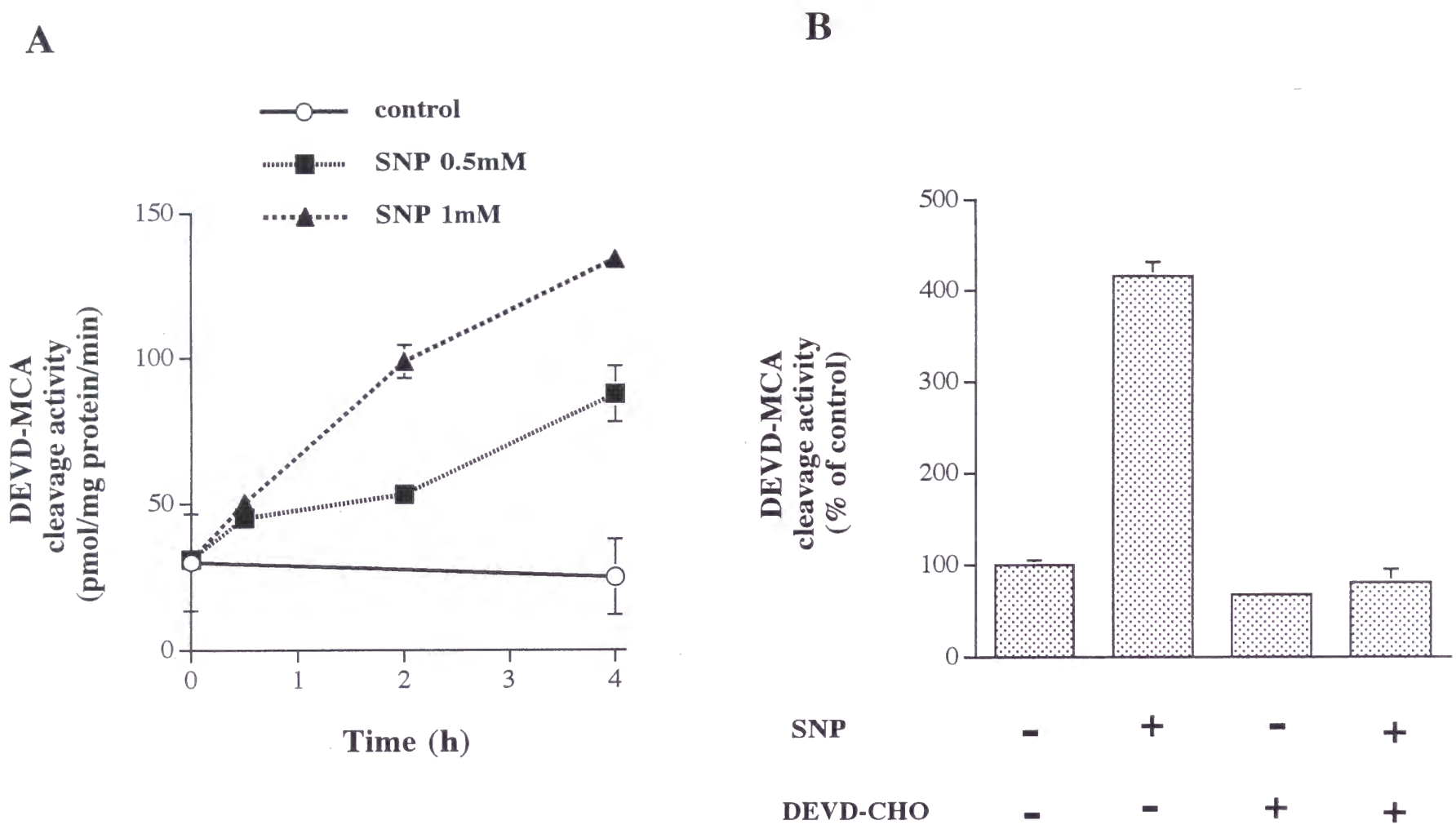

A
control
SNP 0.5mM
SNP 1mM
DEVD-MCA cleavage activity (pmol/mg protein/min)
150
100
50
0
0
1
2
3
4
Time (h)
B
DEVD-MCA cleavage activity (% of control)
500
400
300
200
100
0
SNP
-
+
-
+
DEVD-CHO
-
-
+
+

**Fig.4**

C

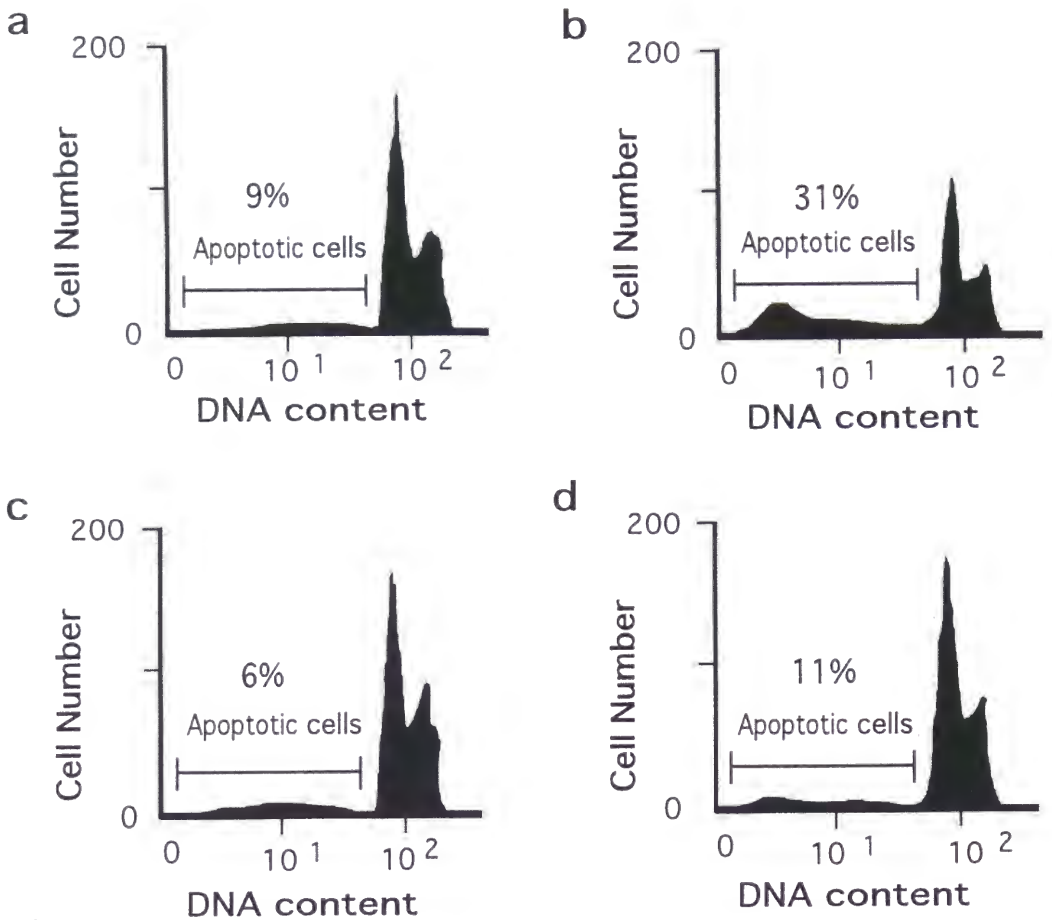

a
200
Cell Number
9%
Apoptotic cells
0
0
10 1
10 2
DNA content
b
200
Cell Number
31%
Apoptotic cells
0
0
10 1
10 2
DNA content
c
200
Cell Number
6%
Apoptotic cells
0
0
10 1
10 2
DNA content
d
200
Cell Number
11%
Apoptotic cells
0
0
10 1
10 2
DNA content

Fig.5

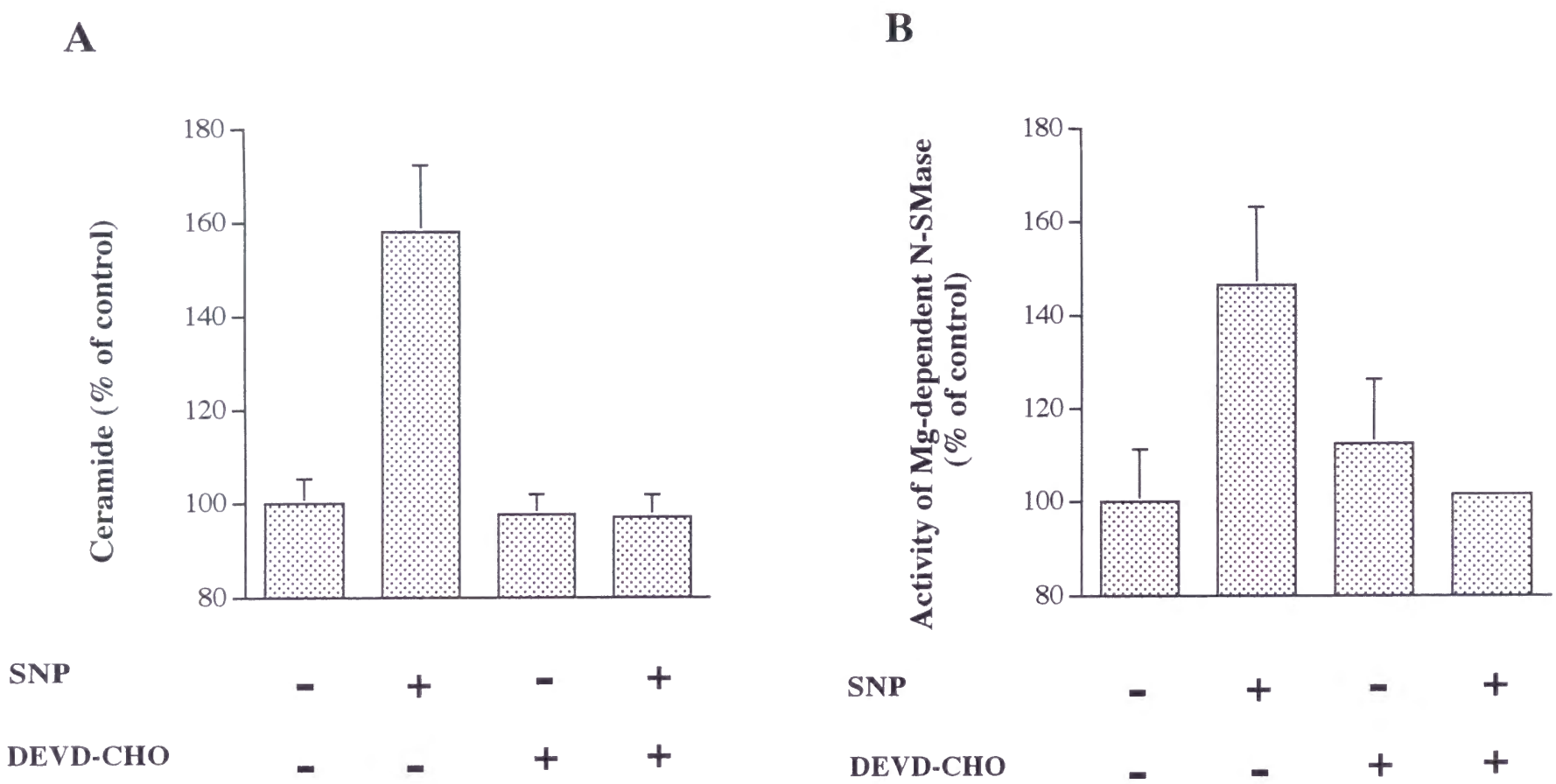

A
Ceramide (% of control)
180
160
140
120
100
80
SNP
- + - +
DEVD-CHO
- - + +
B
Activity of Mg-dependent N-SMase
(% of control)
180
160
140
120
100
80
SNP
- + - +
DEVD-CHO
- - + +

**Fig.6**

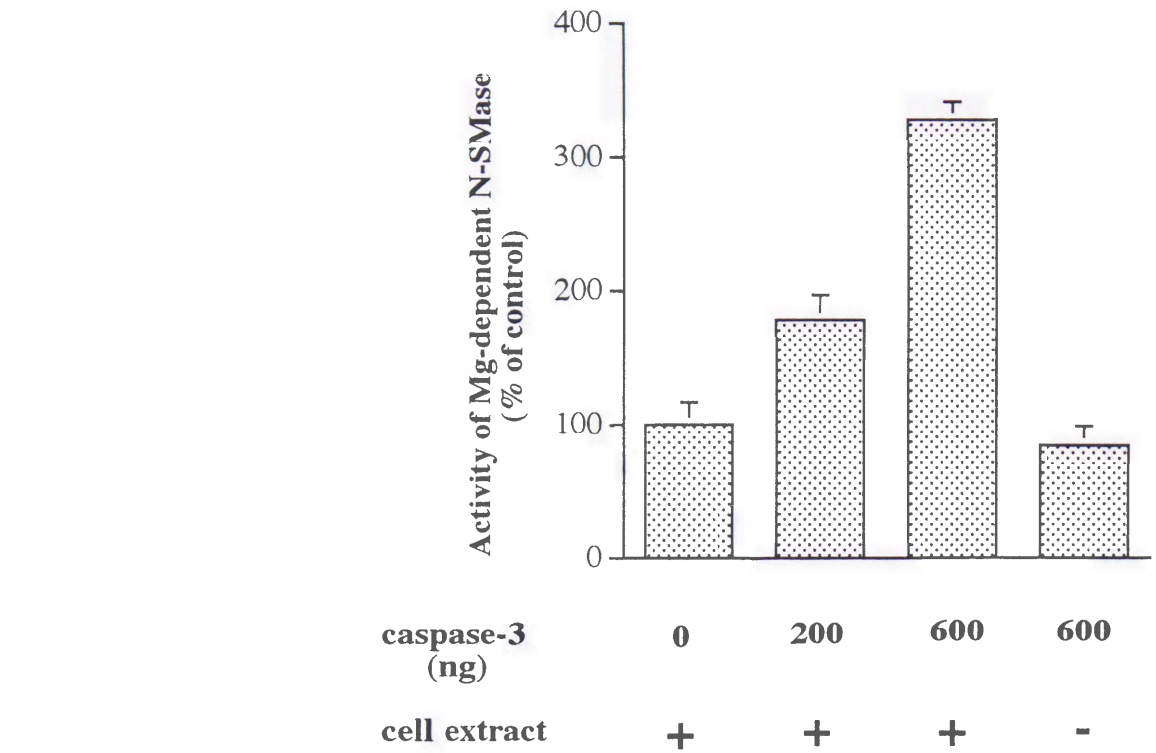

Activity of Mg-dependent N-SMase
(% of control)
400
300
200
100
0
caspase-3
(ng)
0
200
600
600
cell extract
+
+
+
-

FIGURE LEGENDS

FIG. 1 **SNP-induced apoptosis in human leukemia HL-60 cells.** A, time-dependence of apoptotic induction by sodium nitroprusside (SNP). The cells ($2 \times 10^5$/ml) were treated with 1 mM SNP and harvested at the indicated times. Apoptotic cells were determined by FACS analysis using PI staining method as mentioned under "Materials and Methods". B, dose-dependence of apoptotic induction by SNP. The cells were treated with indicated concentrations of SNP for 4 h. The results were obtained from at least three different experiments. The bars indicate one S.D. C, effects of SNP on morphological changes and DNA fragmentation. Left images show the morphological change of the cells without (upper) or with (lower) 1 mM SNP for 3 h. The cells were applied to uncoated glass microscope slides using cytocentrifugation and then stained with May-Giemsa. Right image shows SNP-induced DNA fragmentation 0, 90 and 180 min after treatment with 1 mM SNP. Analysis of DNA fragmentation in agarose gel was performed as described under "Materials and Methods." The results were representative of three different experiments.

FIG. 2 **Changes of nitrite concentration in culture media after treatment with SNP or C2-ceramide.** Nitrite concentration was determined by Griess reaction method as described under "Materials and Methods." Although nitrite increased after treatment with 1 mM SNP as a positive control, no change was observed after treatment with 5 $\mu$M C2-ceramide. The bars indicate one S.D.

FIG. 3 **Ceramide generation, decrease of sphingomyelin and increase of Mg-dependent N-SMase activity by SNP.** A, time-dependent increase of ceramide by SNP. Lipids were extracted at the indicated times and ceramide contents were measured by DGK method as described under "Materials and Methods." The results were obtained from three different experiments. The bars indicate one S.D. B, dose-dependent increase of ceramide by SNP. The cells were exposed to the indicated concentrations of SNP for 4 h. The results were obtained from at least three different experiments. The bars indicate one S.D. C, decrease of sphingomyelin by SNP. The cells prelabeled with [$^{14}$C]choline chloride were treated with 1 mM SNP for the indicated times. Lipids were extracted and labelled sphingomyelin was measured as described under "Materials and Methods." D, SNP-induced activation of Mg-dependent N-SMase. The cells were treated with 1 mM SNP for the indicated times and the activities of SMases were determined as mentioned under "Materials and Methods." The results were obtained from three different experiments. The bars indicate one S.D.

FIG. 4 **SNP-induced activation of caspase and prevention of its activation and apoptosis by DEVD-CHO.** A, induction of DEVD-MCA cleavage activity after treatment with SNP. After the indicated periods of incubation with 0.5 or 1 mM SNP, cleavage activity was measured as described under "Materials and Methods." B, inhibition of SNP-induced caspase by DEVD-CHO. After preincubation for 1 h in the presence or absence of 200 $\mu$M DEVD-CHO, the cells were treated with or without 1 mM SNP for 4 h. C, inhibition of SNP-induced apoptosis by DEVD-CHO. After preincubation for 1 h in the absence or presence of 200 $\mu$M DEVD-CHO, the cells were

treated with or without 1 mM SNP for 4 h (control (a); SNP (b); DEVD-CHO (c); DEVD-CHO + SNP (d)). Apoptotic cells were determined by FACS analysis. The results were representative of three different experiments.

FIG. 5 **Prevention of SNP-induced ceramide generation and Mg-dependent N-SMase activity by DEVD-CHO.** A, the cells were preincubated for 1 h in the absence or presence of 200 $\mu$M DEVD-CHO, and treated for 4 h with or without 1 mM SNP. Lipids were extracted and ceramide contents were determined by DGK assay as described under "Materials and Methods." B, after 1 h preincubation in the absence or presence 200 $\mu$M DEVD-CHO, the cells were incubated with or without 1 mM SNP for 4 h. The activity of Mg-dependent N-SMase was measured as mentioned under "Materials and Methods." The results were obtained from three different experiments. The bars indicate one S.D.

FIG. 6 **Activation of Mg-dependent N-SMase by recombinant purified caspase-3 in cell free system.** In the presence or absence of the cell extract (250 $\mu$g), the assay for Mg-dependent N-SMase was performed with or without the indicated doses of purified caspase-3. Basal specific activity of SMase was 1.29 nmol/mg/h. The results were obtained from three different experiments. The bars indicate one S.D.